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Atty. Docket No. TAK03 P-323

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Art Unit	:	1651
Examiner	:	Francisco C. Prats
Appln. No.	:	10/018,770
Applicants	:	Yoshihito Ikeda et al.
Filing Date	:	December 17, 2001
Confirmation No.	:	2012
For	:	DRUG COMPOSITION CONTAINING A LECITHIN-MODIFIED SUPEROXIDE DISMUTASE

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

APPEAL BRIEF (37 CFR §41.37)

This brief is in furtherance of the Notice of Appeal, filed in this case on July 28, 2005.

The fees required under 35 USC 41(a)(6), and any required petition for extension of time for filing this brief and fees therefor, are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

This brief contains these items under the following headings, and in the order set forth below (37 CFR §41.37(c)):

I. Real Party in Interest

II. Related Appeals and Interferences

III. Status of Claims

IV. Status of Amendments

V. Summary of Claimed Subject Matter

VI. Grounds of Rejection to Be Reviewed on Appeal

VII. Argument

VIII. Conclusion

Appendix of Claims Involved in the Appeal

Evidence Appendix

Related Proceedings Appendix

The final page of this brief bears the attorney's signature.

I. Real Party in Interest

The real party in interest in this application is LTT Bio-Pharma Co., Ltd., the assignment to which was recorded at Reel 014723, Frame 0283.

II. Related Appeals and Interferences

There are not any related appeals or interferences which will directly affect, or be directly affected by, or having a bearing on, the Decision of the Board of Patent Appeals and Interferences.

III. Status of Claims

This is an appeal from the rejection of claims 1, 4, 6-8, 10-14 and 19. Claims 2, 3, 5, 9 and 15-18 have been canceled. No claims have been allowed or withdrawn from consideration.

IV. Status of Amendments

There have not been any amendments filed after the Final Rejection being appealed.

V. Summary of Claimed Subject Matter

Independent claim 1 is directed to a drug composition comprising sucrose and a lecithin-modified superoxide dismutase. Details regarding the meaning of a lecithin-modified superoxide dismutase (also known as phosphatidylcholine-modified superoxide dismutase or PC-SOD) are provided at page 3, lines 1-12 of the specification, and from page 6, line 4 through page 11, line 5 of the specification.

VI. Grounds of Rejection to Be Reviewed on Appeal

Claims 1, 4, 6-8, 10-14 and 19 stand rejected under 35 U.S.C. §103(a) as being unpatentable over JP 9-117279 in view of JP-304882.

VII. Argument

The claims encompass a drug composition comprising a lecithin-modified superoxide dismutase (also referred to as phosphatidylcholine-modified superoxide dismutase or PC-SOD) and sucrose.

The JP '279 document is relevant only to the extent that it discloses PC-SOD, and its potential uses and advantages in pharmacological therapeutic treatments. The JP '279 document does not teach or suggest a need for stabilizing PC-SOD, thus suggesting that PC-SOD does not require stabilization.

The JP '882 translation makes numerous and unambiguous references to the use of stabilizing agents for preventing dimerization of SOD (not PC-SOD). For example, at page 3, the JP '882 translation reads as follows:

> No decrease in the enzymatic action of human SOD is observed when this protein is subjected to freezing and thawing or freeze-drying processes, nor is formation of insoluble matter visible to the naked eye. However, human SOD subjected to analysis by sodium dodecyl sulfate - polyacrylamide electrophoresis, high-performance gel filtration liquid chromatography, and the like produces by-products consisting mostly of dimers.
>
> Thus, the use of human SOD in pharmaceutical products necessitates its stable storage. However, by-products resulting from the storage process may have allergenic side effects; the generation of such substances must be prevented.

This clearly indicates that the authors did not observe any loss of activity, such as may be attributable to denaturation, but observed the formation of dimers, which apparently are only undesirable because they are believed to have an allergenic side effect.

Consistent with the above-quoted statements are the results set forth in Tables 1 and 2 (pages 11 and 17, respectively) which show an absence of denaturation during as many as 10 freeze-thaw cycles of an SOD-containing composition, regardless of whether the composition contained a stabilizer (sorbitol, inositol, sucrose, trehalose or maltose), or no stabilizer at all

(Comparative Example 1). However, the results of Table 1 show that while the stabilizers did not affect denaturation, they provided some reduction in the amount of 79 KD material observed, which the authors regarded as a dimer of SOD. Similarly, the results shown in Table 2 indicate that sorbitol (Embodiment 6), mannitol (Embodiment 7), inositol (Embodiment 8), sucrose (Embodiment 9), trehalose (Embodiment 10), maltose (Embodiment 11), lactose (Embodiment 12), and fructose (Embodiment 13) reduce the amount of 79 KD SOD material formed during freeze-drying of SOD as compared with a similar composition that does not contain a stabilizer (Comparative Example 2). Each of these stabilizers reduces 79 KD product formation, without causing denaturation. In contrast, Comparative Examples 3-5 show that arabinose, glucose and galactose, while also reducing the formation of 79 KD SOD product, cause denaturation. Tables 1 and 2 from the JP '882 translation indicate that denaturation of human SOD does not occur during repeated (up to 10 cycles) freezing and thawing or freeze-drying, except possibly when certain stabilizers outside the scope of the invention disclosed therein (e.g., arabinose, glucose and galactose) are employed. There is some apparent ambiguity on this issue, since the description of the Comparative Examples 3-5 each state "DEAE analysis did not reveal any denaturation of human SOD." However, these statements are inconsistent with the tables, and are also inconsistent with the statement at page 4, second full paragraph of the JP '882 translation which reads as follows:

> However, even with the combination of aldose monosaccharides such as galactose, arabinose, glucose, and the like to human SOD prior to freeze-drying, analysis by anion-exchange chromatography reveals denaturation (Comparative Example 3). Thus, the use of aldose monosaccharides to prevent denaturation of human SOD during freezing or freeze-drying is undesirable.

The results listed under "Test Method (3) Denaturation" in Tables 1 and 2 were determined by anion-exchange chromatography (see page 8, second full paragraph, which indicates that analysis by anion-exchange chromatography is referred to as DEAE analysis). Thus, the above-quoted language clearly refers to Comparative Examples 3-5. When properly understood with reference to the disclosure in its entirety, the tabulated data suggest that denaturation of human SOD does not occur unless a material which causes denaturation is added. Clearly, the JP '882

translation is teaching that lactose, arabinose and glucose cause denaturation of human SOD, which would not otherwise occur during freezing or freeze-drying.

The Examiner has taken the position that "the use of sucrose as a protein stabilizing agent for PC-SOD is considered obvious, in view of the art-recognized stabilizing effect of sucrose on proteins, including SOD, in storage processes using lyophilization (i.e., freeze-drying)."

The sole issue in this application can be resolved by determining whether a prior art disclosure to add sucrose to SOD for preventing or reducing denaturation (unfolding of the SOD) and/or dimerization (alleged by the prior art to have allergenic side effects) would motivate one having ordinary skill in the art to add sucrose to phosphatidylcholine-modified SOD (PC-SOD).

There is an absence of any suggestion in the prior art that PC-SOD is susceptible to denaturation, that dimerization of PC-SOD is undesirable, or that PC-SOD dimers have allergenic side effects. To the contrary, U.S. Patent No. 5,762,929 (Exhibit 1) discloses that PC-SOD is "useful as an anti-inflammatory agent without adverse effect such as antigenicity" (column 1, lines 59-61). This means that PC-SOD does not exhibit allergenic side effects, although it already "is a homodimer" (see column 7, line 38 of U.S. Patent No. 5,762,929 (Exhibit 1)). Thus, the motivation disclosed in the JP '882 translation for adding a stabilizer such as sucrose to SOD is not present with PC-SOD.

While the JP '882 translation indicates that bovine SOD (not PC-SOD) is susceptible to denaturation during freeze-drying, those having ordinary skill in the art would not expect PC-SOD to undergo denaturation during freeze-drying, and may actually expect that the addition of sucrose could cause denaturation. Lindberg et al., "Folding of human superoxide dismutase: Disulfide reduction prevents dimerization and produces marginally stable monomers," *Proceedings of the National Academy of Sciences of the United States of America*, November 9, 2004, Vol. 101, No. 45 (Exhibit 2) teach (see the abstract) that there is a kinetic relationship between the denaturated (uncoiled) SOD monomer (D) and the folded monomers (M), and another kinetic relationship between the folded monomers (M) and the SOD dimer (M_2). This

indicates that denaturation of SOD dimers cannot occur unless each of the individual dimers is first dissociated into two folded monomers (M). The JP '882 translation suggests that sucrose prevents dimerization of SOD, which necessarily suggests that the addition of sucrose to SOD will drive the kinetics toward dissociation of dimers (M_2) into folded monomers (M) that can subsequently be converted to denatured monomers (D). Thus, one skilled in the art would not be motivated to add sucrose to PC-SOD, since therapeutically active PC-SOD is a homodimer (a molecule comprised of two identical polypeptide chains) that does not exhibit antigenicity, and because the prior art teaches that denaturation of SOD dimers cannot occur unless the SOD is first dissociated into monomers.

It should be noted that U.S. Patent No. 5,762,929 (Exhibit 1) discloses (Example 2, column 11, line 46 through column 12, line 3) tablets and capsules prepared with lyophilized PC-SOD, without suggesting that there is any need for adding a stabilizer of any type during freeze-drying of the PC-SOD (lactose is added to the tablet as an excipient along with the already lyophilized PC-SOD). The application for U.S. Patent No. 5,762,929 (Exhibit 1) was based on a Japanese application filed January 31, 1995, well after the August 12, 1989 publication date of the JP '882 application. This strongly suggests that while there have been well known stability issues with SOD, there are no similar stability issues with PC-SOD that would suggest utilization of stabilizers.

Those having ordinary skill in the art of preparing pharmaceutical compositions for the treatment of disease are typically very well educated and possess a comprehensive knowledge of organic chemistry and biochemistry. A person of ordinary skill in the pertinent art would typically have an advanced degree in chemistry, molecular biology and/or pharmacology, would be fully cognizant of the technology described in the prior art, and would be capable of understanding the underlying chemistry. Thus, a person of ordinary skill in the art would understand that lecithin-modified superoxide dismutase (PC-SOD) is a chemical substance that is different from SOD. It is well known that PC-SOD differs significantly from conventional SOD with respect to distribution within the living body and affinity to self. It is also well known that

PC-SOD retains an extremely uniform activity as compared with SOD, so that it is expected to enhance the pharmacological activity of SOD, reduce side effects, and promote absorption.

Because it is well known that PC-SOD is chemically very differently from SOD, those having ordinary skill in the art would not expect sucrose to have the same stabilizing effect on PC-SOD as it does on SOD. In fact, it would be contrary to the teachings of the prior art to shift the kinetics and equilibrium toward monomeric PC-SOD and one step closer to denatured (uncoiled) PC-SOD (assuming, as the Examiner has, that PC-SOD and SOD exhibit similar chemical properties).

It is respectfully submitted that the rejection is not supported by the prior art, but is instead based on speculation and hindsight. It is based on speculation that those having ordinary skill in the art would expect allergenic side effects due to dimerization of PC-SOD, similar to the alleged problems associated with dimerization of SOD, despite the fact that the prior art (Exhibit 1) teaches that therapeutically active PC-SOD already exists as a dimer and does not exhibit allergenic side effects. It is further based on speculation that PC-SOD would be expected to undergo denaturation during freeze-drying, despite the fact that there is no indication in the prior art that PC-SOD is susceptible to denaturation and that the prior art only discloses denaturation during freeze-drying of <u>bovine SOD</u>, not PC-SOD or human SOD, with PC-SOD being at least as different chemically from SOD as bovine SOD is from human SOD (e.g., according to the JP '882 translation, bovine SOD is susceptible to denaturation during freeze-drying, whereas human SOD is not). There is no reasonable basis for concluding from the prior art that there is a need for adding a stabilizer such as sucrose to protect PC-SOD against degradation of any type. In fact, the prior art (Exhibit 2) suggests that sucrose might bring the PC-SOD dimer one step closer to denaturation.

Appellants have discovered that there is a loss of biological activity of PC-SOD during freeze-drying and/or freeze-thaw cycles due <u>only</u> to degradation of the phosphatidylcholine (PC) moieties. Appellants discovered this problem and a solution to this problem without any guidance from the prior art. It is only by utilizing Appellants' own teachings as a guide that one

of ordinary skill in the art could misinterpret and extrapolate the teachings of the prior art to piece together Appellants' invention.

VIII. Conclusion

Upon consideration of the applied prior art references and applicable law, Appellants submit that reversal of the rejection is appropriate and is requested.

Respectfully submitted,

September 7, 2005
Date

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Appendix of Claims (35 USC §41.37(c)

1. A drug composition comprising sucrose and a lecithin-modified superoxide dismutase represented by the following general formula (I):

$$SOD'(Q\text{-}B)_m \qquad (I)$$

wherein SOD' is a residue of superoxide dismutase; Q is a chemical crosslinking; B is a residue without a hydrogen atom of a hydroxyl group of lysolecithin having the hydroxyl group at the 2-postion of glycerol; m is an average number of bonds of lysolecithin to one molecule of superoxide dismutase which is a positive number of 1 of more.

4. The drug composition according to claim 1 wherein a fatty acid content in the drug composition is 0.13-0.15 μmol/mg protein.

6. The drug composition according to claim 1 or 4 wherein Q is $-C(O)-(CH_2)_n-C(O)-$, n being an integer of 2 or more.

7. The drug composition according to claim 1 or 4 wherein SOD' is a residue of human superoxide dismutase.

8. The drug composition according to claim 1 or 4 wherein SOD' is a residue of a modified form of superoxide dismutase in which an amino acid in 111-position of an amino acid sequence of human superoxide dismutase is converted into S-(2-hydroxyethylthio) cysteine.

10. The drug composition according to claim 1 or 4 wherein n is an integer of 2 to 10.

11. The drug composition according to claim 1 or 4 wherein m is a positive number of 1 to 12.

12. The drug composition according to claim 1 or 4 wherein the sucrose has been treated with activated charcoal.

13. The drug composition according to claim 1 or 4 wherein the drug composition is lypholized.

14. The drug composition according to claim 1 or 4 wherein a weight ratio of the lecithin-modified superoxide dismutase to sucrose is 0.4/100-60/100.

19. A composition containing lecithin-modified superoxide dismutase that is reconstituteable from a dry form and which has been stabilized against degradation due to cleavage within the lecithin moieties, comprising:

lyophilized lecithin-modified superoxide dismutase; and

sucrose in an amount that is effective to stabilize the lecithin-modified superoxide dismutase against degradation due to cleavage within the lecithin, whereby there is not any observable difference in the amount of degradation products before lyophilization and after re-dissolution, and wherein the composition completely dissolves in water in less than 10 seconds.

Evidence Appendix (35 USC §41.37(c))

The following evidence submitted during prosecution of this application are relied upon by Appellants in this appeal:

Exhibit1: U.S. Patent No. 5,762,929

Exhibit 2: Lindberg et al., "Folding of human superoxide dismutase: Disulfide reduction prevents dimerization and produces marginally stable monomers," *Proceedings of the National Academy of Sciences of the United States of America*, November 9, 2004, Vol. 101, No. 45.

Related Proceedings Appendix (35 USC §41.37(c))

There have not been any related appeals or interferences during pendency of this application.

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BIOCHEMISTRY

Folding of human superoxide dismutase: Disulfide reduction prevents dimerization and produces marginally stable monomers

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Edited by Peter G. Wolynes, University of California at San Diego, La Jolla, CA, and approved September 23, 2004 (received for review June 4, 2004)

Abstract

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The molecular mechanism by which the homodimeric enzyme Cu/Zn superoxide dismutase (SOD) causes neural damage in amytrophiclateral sclerosis is yet poorly understood. A striking, as well as an unusual, feature of SOD is that it maintains intrasubunit disulfide bonds in the reducing environment of the cytosol. Here, we investigate the role of these disulfide bonds in folding and assembly of the SOD apo protein (apoSOD) homodimer through extensive protein engineering. The results show that apoSOD folds in a simple three-state process by means of two kinetic barriers: $2D \rightleftharpoons 2M \rightleftharpoons M_2$. The early predominant barrier represents folding of the monomers (M), and the late barrier the assembly of the dimer (M_2). Unique for this mechanism is a dependence of protein concentration on the unfolding rate constant under physiological conditions, which disappears above 6 M Urea where the

transition state for unfolding shifts to first-order dissociation of the dimer in accordance with Hammond-postulate behavior. Although reduction of the intrasubunit disulfide bond C57–C146 is not critical for folding of the apoSOD monomer, it has a pronounced effect on its stability and abolishes subsequent dimerization. Thus, impaired ability to form, or retain, the C57–C146 bond *in vivo* is predicted to increase the cellular load of marginally stable apoSOD monomers, which may have implications for the amytrophic lateral sclerosis neuropathology.

protein folding | protein stability | disulfide bond | transition-state shifts | protein engineering

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The mechanism by which mutant superoxide dismutase (SOD) leads to neural damage in the familial form of amyotrophic lateral sclerosis (ALS) is yet unknown (1–3). In analogy with other neurodegenerative disorders (4), however, an increasing body of observations (5–16) suggest that the ALS disease mechanism is coupled to destabilization or misfolding of the SOD structure (17), manifested ultimately by cellular inclusions of SOD aggregates (3, 18). From a strictly energetic perspective, the native SOD structure is distinctive by containing one oxidized disulfide bond per monomer (C57–C146) in the reducing environment of the cytosol (19) (Fig. 1). Normally, disulfide bonds are maintained only under oxidizing conditions in the extracellular space (20, 21) where they increase protein stability by confining the configurational entropy of the denatured ensemble (22). Indications that the integrity of the C57–C146 bond may have bearing on the molecular events in ALS were recently provided by Tiwari and Hayward (15), who demonstrated that ALS-associated SOD mutants are more susceptible to chemical disulfide reduction than the wild-type protein. More detailed elucidation of the structural and energetic effects accompanying mutational perturbations and disulfide reduction has so far been prevented by scarce knowledge about how the SOD homodimer folds (23).

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Fig. 1. Structural comparison of the SOD homodimer and monomer. (*Left*) Structure of the human SOD homodimer (1SPD[PDB]), showing the positions of the cysteines, the loop containing residues 49–84, and the Cu and Zn ions. (*Right*) Loop displacements upon dissociation of the SOD homodimer (illustrated on the right-hand-side monomer), obtained by structural superposition of holoSOD^{wt} (blue) (51) and holoSOD$^{50/51/E133Q}$ (orange) (41).

In this study, we shed further light on this issue by mapping out the folding and assembly reaction of metal-depleted SOD through kinetic and thermodynamic analysis of mutant protein, with particular focus on the role of the intrasubunit disulfide bond C57–C146. After elimination of artifactual cross-linking by substitution of the solvent-accessible cysteines 6 and 111, the oxidized SOD apoenzyme (apoSOD) homodimer reveals a conspicuous chevron plot indicative of a three-state process in which the rate-limiting step shifts from the first-order nucleation of the monomers at low-urea concentrations, to the transition state for monomer dissociation at high-urea concentrations. A benefit of this transition-state change is that information about monomer folding and dimer formation can be derived from kinetic data alone. The results show that the C57–C146 bond only marginally affects the folding process of the monomeric protein, but is crucial for its stability and dimerization in the absence of Cu and Zn.

Materials

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Proteins. The single and multiple SOD mutations C6A, C111A, C6A/C111A, and C6A/C111A/C57A/C146A and F50E/G51E (monomeric) variants thereof were constructed as described in ref. 12. Samples of apoSOD (molecular mass of 32 kDa for dimer) were prepared in two ways: (*i*) on a chelate column under mildly acidic conditions (pH 3.5) according to the procedures in ref. 24 and dialyzed against 10 mM EDTA at pH 7; and (*ii*) by unfolding in 4 M guanidinium chloride in the presence of 50 mM EDTA and then refolding by dialysis against 10 mM Mes (pH 6.3) with 10 mM EDTA (standard buffer). Reducing conditions were obtained by the addition of 2 mM Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) in the unfolding buffer and 0.5 mM TCEP·HCl in all other steps.

Measurements. Equilibrium titrations were done on a J-810 CD spectrometer (Jasco, Easton, MD) or a 4*-180 instrument (Applied Photophysics, Surrey, U.K.), and the kinetic measurements were carried out on an SX-17MV stopped-flow fluorimeter (Applied Photophysics) at 25°C. Protein concentration was 4 μM or 70 μM monomer.

Data Analysis. In the kinetic analysis, monomeric apoSOD was assumed to display a minimal two-state transition between the denatured state (D) and the fully folded monomer (M), $K_{D-M} = [D]/[M] = k_u/k_f$:

$$\log K_{D-M} = \log K_{D-M}^{H_2O} + m_{D-M}^{\log K}[\text{urea}], \quad [1]$$

$$\log k_u = \log k_u^{H_2O} + m_u[\text{urea}], \text{ and} \quad [2]$$

$$\log k_f = \log k_f^{H_2O} + m_f[\text{urea}], \quad [3]$$

where the slopes $m_{D-M}^{\log K} = m_u - m_f$ are commonly taken as a measure of solvent exposure in the equilibrium unfolding process and the activation process of unfolding and refolding, respectively (25). The parameters were derived from equilibrium data by using the equation

$$K_{D-M} = [D]/[M] = f_D/(1 - f_D), \quad [4]$$

where f_D is the fraction of D, and protein stability was derived as $\Delta G_{D-M} = -RT \ln K_{D-M} = \Delta G_{D-M}^{H_2O} + m_{D-M}^{\Delta G}[\text{urea}]$. The plots of log k_f and log k_u vs. [urea] were fitted by

$$\log k_{obs} = \log(k_f + k_u) = \log(10^{\log k_f^{H_2O} + m_f[\text{urea}]} + 10^{\log k_u^{H_2O} + m_u[\text{urea}]}) \quad [5]$$

and the position of the transition-state ensemble was obtained from

$$\beta^\dagger = -m_f/m_{D-N} = m_f/(m_f - m_u). \quad [6]$$

The ϕ-values were calculated from wild-type and mutant data by the standard relation (26)

$$\phi = \Delta\Delta G^{\ddagger}/\Delta\Delta G_{D-N} = \Delta \log k_f/(\Delta \log k_f - \Delta \log k_u), \quad [7]$$

where $\Delta\Delta G^{\ddagger}$ and $\Delta\Delta G_{D-N}$ are the mutant-induced destabilization of the transition-state ensemble and the folded structure, respectively. A ϕ-value of 1 suggests that the structure around the mutated residue is native-like in the transition state ensemble, whereas a ϕ-value of 0 suggests that it is unfolded. Fractional ϕ-values indicate that the targeted region is partly structured in the transition state ensemble.

The stability of dimeric apoSOD, $\Delta G_{2D-M2} = -RT \ln K_{2D-M_2}$, was derived from equilibrium denaturation data according to

$$K_{2D-M_2} = [D]^2/[M_2] = 2P f_D^2/(1 - f_D), \quad [8]$$

assuming linear free-energy dependence on [urea]. Data analysis was done by using the KALEIDAGRAPH software (Synergy Software, Reading, PA).

Results

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Disulfide Reduction Leads to Dissociation of the apoSOD Dimer. The dimer integrity of oxidized and reduced SOD was analyzed by gel chromatography on a Sephadex S-75 column (Pharmacia). To minimize the effects of local charge differences between the holo- and apo-species, 150 mM NaCl was added to the standard buffers. The concentration of the eluted protein was ≈20 μM monomer. Oxidized apoSOD^{wt} elutes at 10.5 ml, corresponding to the dimeric species (Fig. 2*a*). This peak is well separated from that of the monomeric double mutant F50E/G51E (apoSOD$^{50/51}$) at 12 ml (Fig. 2). Upon reduction of the cysteines, apoSOD^{wt} elutes mainly as a folded monomer with only a small residual of dimers. For comparison, holoSOD^{wt} remains dimeric in both its oxidized and disulfide-reduced state (data not shown). The results suggest that assembly of the SOD homodimer requires either the oxidized disulfide linkage between Cys-57 and Cys-146, or coordinated metal ions; the absence of both promotes dissociation. Consistently, the fully cystein-depleted mutant C6A/C111A/C57A/C146A (SOD^{CallA}) elutes as a folded monomer in its apo state and as a dimer with coordinated metals, although the dimeric state of this radical construct travels somewhat slower than the wild-type ditto (Fig. 2). The apo state of the double mutant C6A/C111A (apoSOD$^{6/111}$), in which only the solvent-accessible cysteines have been replaced, monomerizes upon disulfide reduction in a manner identical to that of the wild-type protein (Fig. 2).

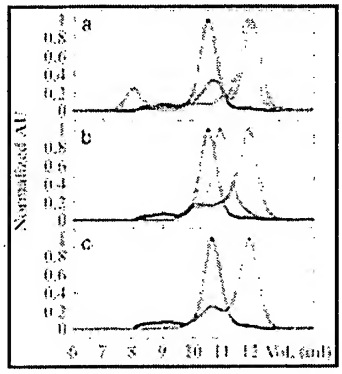

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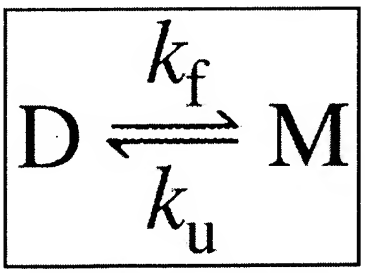
Fig. 2. Size-exclusion chromatograms showing the dimer/monomer composition of wild-type and mutant SOD with and without the C57–C146 disulfide linkage. Dimers elute at 10–11 ml and folded monomers at 12 ml. (*a*) Oxidized apoSOD^{wt} (black), reduced apoSOD^{wt} (red), and oxidized apoSOD$^{50/51}$ (blue). (*b*) Oxidized apoSOD^{wt} (black), fully cysteine-depleted apoSOD (red), and fully cysteine-depleted holoSOD (blue). (*c*) Oxidized apoSOD$^{6/111}$ (black) and reduced apoSOD$^{6/111}$ (red). AU, absorbance unit.

Monomeric SOD Shows Classical Two-State Folding Behavior. Equilibrium denaturation of the reduced apoSOD^{wt} monomer by urea reveals a sigmoidal unfolding transition consistent with a two-state process (see Scheme 1), where D is the denatured ensemble and M is the folded monomer (data not shown). Fitting of Eq. **4** yields a midpoint of [urea] = 1.51 M and $m_{D-M} = 1.42\ M^{-1}$, corresponding to $\log K_{D-M}^{H_2O} = 2.14$ or a stability of $\Delta G_{D-M}^{H_2O} = 2.91$ kcal/mol in the absence of urea (Eq.**1** and Table 1). Compared with other single-domain proteins, this stability is notably low (Fig. 4). Additional evidence for two-state folding is provided by the kinetics. The chevron plot of apoSOD^{wt}, i.e., a plot of log k_f and log k_u vs. urea, is characteristically v-shaped with slopes $m_f = -1.05\ M^{-1}$ and $m_u = 0.48\ M^{-1}$ (Eqs. **2**, **3**, and **5**) that sum up to $m_{D-M}^{\log K} = 1.5\ M^{-1}$ from equilibrium data (Fig. 3 and Table 1). Moreover, the sum of $\log k_f^{H_2O}$ and $\log k_u^{H_2O}$ matches $\log K_{D-M}^{H_2O}$, yielding the relation $K_{D-M} = k_u/k_f$. By these criteria, the monomeric state of apoSOD^{wt} classifies as a "classical" two-state folder (26). That is, monomeric apoSOD^{wt} describes a highly concerted transition over a pointed free-energy barrier, in accord with the majority of other single-domain proteins (27). The position of the transition-state ensemble on this barrier profile is $\beta^{\ddagger} = 0.69$ (Eq. **6**), in good correspondence with the values observed for other two-state folders (27). The reduced state of the monomeric variant apoSOD$^{50/51}$ displays overall an identical chevron plot but with a slightly decreased value of $\log k_f^{H_2O}$ (Fig. 3 and Table 1). Although this small decrease of the refolding rate constant ($\Delta \log k_f^{H_2O} \approx -0.2$) is a notable and consistent feature of the F50E/G51E mutation, it is nevertheless too small to permit structural interpretation by ϕ-value analysis (28).



Scheme 1.

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Table 1. Kinetic and thermodynamic parameters for wild-type and mutant apoSOD

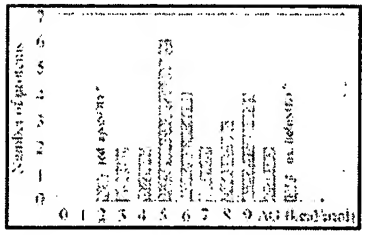

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Fig. 4. Stability histogram for 27 single-domain proteins adapted from ref. 27. The apparent stabilities of the reduced apoSOD^{wt} monomer and the native holoSOD^{wt} dimer are found on the extreme sides of the distribution.

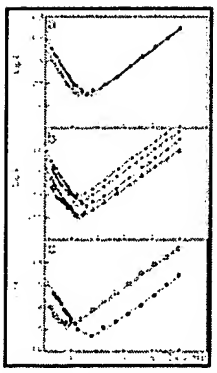

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Fig. 3. Chevron plots of wild-type and mutant SOD monomers display simple two-state folding behavior. Units are in s^{-1}. (*a*) Reduced apoSOD^{wt} (●) and reduced apoSOD$^{50/51}$ (○). (*b*) Reduced apoSOD^{wt} (●), reduced apoSOD^{C6A} (△), reduced apoSOD^{C111A} (▲), and reduced apoSOD$^{6/111}$ (▼). (*c*) Reduced apoSOD$^{50/51/6/111}$ (□) and oxidized apoSOD$^{50/51/6/111}$ (■).

The Role of Cys-6 and Cys-111 in Folding and Stability. Because SOD^{wt} tends to aggregate under conditions promoting disulfide bond formation, we have used the much more controllable variant apoSOD$^{6/111}$, lacking free cysteine residues, as pseudo wild-type in the kinetic analysis of the oxidized protein (29). The procedure is validated as follows. Neither of the single mutations C6A or C111A nor the double mutant C6A/C111A shows any appreciable change of the folding *m*-values, indicating that their influence on the folding trajectory is negligible (25) (Fig. 3 and Table 1). With respect to the energetics, reduced C6A affects mainly the refolding limb of the chevron plot. Truncation of the thiol group destabilizes the transition-state ensemble ($\Delta\Delta G^{\ddagger} = 2.3RT$ $\Delta\log k_f = 0.47$ kcal/mol) and the folded monomer ($\Delta\Delta G_{D-N} = 0.47$ kcal/mol) to a similar extent, yielding a ϕ-value of 1.0 (Eq. 7). Thus, the structure around this position seems to form early in folding and constitutes part

of the critical nucleus in the transition-state ensemble (25). The small decrease of k_u accompanying reduced C6A is rarely seen for truncations of methylene groups but could, in this case, stem from the desolvation penalty of the thiol moiety that is larger in the folded protein than in the more expanded transition-state ensemble. In contrast, the mutation C111A affects mainly the unfolding kinetics, indicative of a more selective destabilization of the folded monomer (Fig. 3 and Table 1). The values of $\Delta\Delta G^{\ddagger} = 0.19$ kcal/mol, $\Delta\Delta G_{D-N} = 0.8$ kcal/mol, and $\phi = 0.24$ suggest that the structural environment of C111 is largely unfolded in the transition state. Finally, the result of reduced apoSOD$^{6/111}$ is simply the sum of the two single mutants, providing further (site-specific) evidence that the substitutions C6A and C111A do not significantly influence the folding trajectory of the apoSOD monomer (Fig. 3 and Table 1). As an additional control, the fully cysteine-depleted quadruple mutant mimics precisely the behavior of reduced apoSOD$^{6/111}$ (Table).

The Intrasubunit Disulfide Linkage Has Minor Influence on Folding but Is Important for Monomer Stability. To investigate the role of the C57–C146 disulfide bond in the early folding events, we used apoSOD$^{50/51/6/111}$, which is the only construct in this study that allows selective detection of monomer unfolding and stability under oxidizing conditions. From the chevron plots in Fig. 3 it is apparent that the main contribution of the C57–C146 disulfide bond is to promote monomer stability by decreasing the unfolding rate constant ($\Delta\Delta G_{D-N} = 1.56$ kcal/mol, Table 1 and Fig. 3). The corresponding effect on the transition-state ensemble is notably smaller at $\Delta\Delta G^{\ddagger} = 0.67$ kcal/mol, yielding an apparent ϕ-value of 0.43 (Table 2). At a molecular level, the interpretation of this ϕ-value is somewhat different from that of side-chain truncations (22). In contrast to the enthalpic contribution of side-chain contacts, disulfide linkages stabilize folded structures by reducing the configurational entropy of disordered states. The fractional effect of the C57–C146 disulfide bond on the apoSOD transition-state ensemble, suggests then that the part of the polypeptide chain that is restricted by this linkage is largely disordered in the rate-limiting step for folding. That is, the contacts anchoring loop around position 57 are not required for the early folding events and consolidate mainly on the downhill side the folding barrier. The result is not surprising because it is expected that the correct topological context needs to be firmly established before disulfide-bond formation in the reducing environment of the cytosol.

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Table 2. Data for the apoSOD$^{6/111}$ dimer

Folding of the apoSOD Homodimer. As expected, the apparent stability of the oxidized apoSOD$^{6/111}$ homodimer increases at increased protein concentration. The transition midpoint shifts from 2.7 M urea at 4 μM apoSOD$^{6/111}$ to 3.1 M urea at 70 μM (Table 2), and the hidden surface area of the dimer interface contributes to an apparent m-value that is ≈0.2 units larger than for the monomer. The values of $\Delta G^{6/111}_{2D-M_2}$, as derived from equilibrium unfolding data at 4 and 70 μM protein (Eq. 8), are −15.8 ± 0.6 kcal/mol and −15.6 ± 0.5 kcal/mol, respectively, in good correspondence with earlier reports on the apo protein (30) (Table 2). The refolding kinetics of dimeric apoSOD$^{6/111}$ resembles closely that of the oxidized monomer apoSOD$^{50/51/6/111}$ (Fig. 5 and Table 1): the values of m_f are within experimental errors, whereas the small offset in logk_f of 0.26 matches that observed for the dimer-splitting substitutions F50E/G51E. Moreover, the refolding rate constant for dimeric

apoSOD$^{6/111}$ shows no dependence on protein concentration: the refolding limbs obtained at 4 μM and 70 μM apoSOD$^{6/111}$ are indistinguishable (Fig. 5 and Table 1). On this basis, we conclude that refolding of the SOD homodimer is rate limited by the first-order formation of the monomer. We note, however, that an additional faster phase emerges at high protein concentrations, whose amplitude constitutes <20% of the total fluorescence change. This minor phase is not resolved with the monomeric protein, and its origin is yet unclear. One possibility is that it stems from transient aggregation of denatured or partly folded protein in the absence of the hydrophilic substitutions F50E/G51E (31). Another possibility is that a subfraction of the SOD molecules assemble before the global refolding transition, or form monomeric intermediates. The major difference between the monomeric and homodimeric protein is seen in the unfolding kinetics: the values of $\log k_u$ are substantially decreased, and the chevron plot shows a pronounced downward kink at around 5 M urea (Fig. 5). Similar kinks have been described for other proteins and are the hallmarks for changes of the rate-limiting step (32, 33); i.e., the transition state shifts closer to the native state along the experimental progress coordinate in accordance with the Hammond postulate (34). In the case of SOD, it is evident that the unfolding reaction proceeds over the transition state for monomer formation at low [urea], to become rate limited by the first-order dissociation of the dimer above 5 M urea (see Scheme 2). In direct support of this folding model, increased protein concentration affects solely the steeper region of the unfolding limb. A qualitative description of the effect is provided by the free-energy profiles in Fig. 5. Below 5 M urea, the unfolding time course is modulated by both the activation barrier for unfolding of M and the second-order equilibrium between M_2 and M. Under these conditions, elevated protein concentrations slow down the unfolding process by shifting the equilibrium toward M_2, i.e., the initial free-energy difference between M_2 and M is increased. Above 5 M urea, the dependence on protein concentration vanishes because the rate-limiting step moves to become the first-order dissociation of M_2. To visualize the dimer folding in a single chevron plot, we have tentatively treated the unfolding kinetics as first-order at all concentrations of denaturant. The second-order component in the unfolding reaction below 5 M urea does not cause any detectable deviation from exponential time courses. The $[M]^2/[M_2]$ equilibrium constant at 0 M urea was estimated from the stability difference between dimeric and monomeric protein, $\Delta G^{6/111}_{2M-M_2} = \Delta G^{6/111}_{2D-M_2} - (\Delta G^{50/51/6/111}_{D-M} - \Delta\Delta G^{50/51}_{D-M})$, where $\Delta\Delta G^{50/51}_{D-M}$ is the adjustment for the mutation F50E/G51E (Table 2). The value of $\Delta G^{6/111}_{2M-M_2} \approx -12$ kcal/mol yields a dissociation constant of 1.5 nM, which is in precise agreement with previous estimates (30).

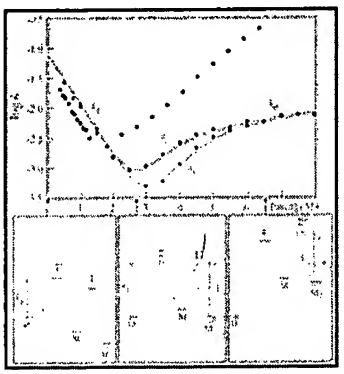

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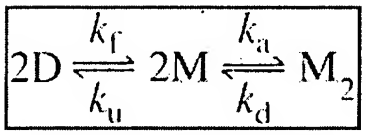
Fig. 5. Chevron plots for the apoSOD$^{6/111}$ dimer at protein concentrations of 4 μM (blue) and 70 μM (red). For comparison, shown are the data for the monomer apoSOD$^{50/51/6/111}$ (black). Units are in s^{-1}. The fits are from a three-state model according to Scheme 2 (52). Below are the corresponding folding free-energy profiles at 0, 2.7, and 6.5 M urea, as estimated from transition-state theory with prefactor of 10^6 s^{-1} (53), and the apparent stabilities $f_D/(1 - f_D)$ of M and M_2 by using chevron and equilibrium data, respectively. At low [urea], the dimeric and monomeric proteins seem to fold over the same transition state (‡), where the offset in $\log k_f$ is due to the F50E/G51E mutation. Above the transition midpoint, $\log k_u$ decreases with increasing protein concentrations as the free-energy difference between ‡ and M_2 goes up.

[in a new window] This concentration dependence vanishes at high [urea] where the rate-limiting step shifts to the first-order dissociation of M2→2M according to Hammond-postulate behavior (33).



Scheme 2.

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Discussion

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Disulfide Reduction Promotes Dimer Dissociation. Compared with other homodimeric proteins, the ratio between interfacial and intramonomer contacts in SOD is relatively small (35). Characteristic for such proteins is that monomer folding precedes dimerization, producing interfaces that are overall less optimized than those of obligatory dimers where folding and association occur in one highly concerted step (35, 36). Consistently, the crystal structures of the ALS mutants A4V and I113T are reported to display marked distortions of the dimer interface although the structural effect on the individual monomers is small (37). Hough *et al.* (37) suggest that the decreased stability of the dimer interface expected to result from this distortion could be an integral part of the ALS disease mechanism. Along the same line, neurodegeneration in Parkinson's disease has been linked to an interface mutation in the homodimeric protein DJ-1 (38), and familial amyloid polyneuropathy has been linked to dissociation of the transthyretin tetramer into alternatively folded monomers (39). In this study, we reveal an additional aspect of the SOD homodimer by showing that its association equilibrium is also critically dependent on the intrasubunit disulfide bond C57–C146. Upon reduction of this disulfide bond, the apo protein completely fails to dimerize at physiological concentrations [see also the recent work by Furukawa *et al.* (40) arriving at the same conclusion]. That is, SOD needs to maintain the C57–C146 bond oxidized under the reducing pressure in the cytosol to ensure full dimer stability. This redox sensitivity is a most unusual property of intracellular proteins that could constitute an Achilles' heel of the SOD molecule. From this perspective, it is interesting to note that ALS-associated mutants exhibit an increased susceptibility to disulfide reduction (15), implicating a scenario where the effect of otherwise benign mutations is augmented by loss of the C57–C146 bond (15). Consistently, we have evidence for the susceptibility of mutant SOD proteins to thioredoxin and thioredoxin reductase, the major disulfide reduction system of the cell responsible for keeping the cytosol reduced (M.O., M.J.L., and A.H., unpublished results).

Loop Dynamics Can Modulate Interface Stability The structural connection between the dimer interface and the disulfide link is by means of the large loop between strands 4 and 5 (Fig.). The loop forms part of the interface (residues 50–54) and is anchored to the β barrel by the C57–C146 bond. It encompasses also the Cu and Zn ions through the coordinating residues H46, H48, H63, H71, H80, and D83 (41). The importance of this

loop in modulating the docking of the two SOD monomers is clearly emphasized by superposition of the crystal structures of the dimeric and monomeric proteins: the loop displays a significant displacement on either side of the disulfide anchoring point in the monomer (41). It is thus plausible that cleavage of the C57–C146 bond favors the monomeric species entropically by simply increasing the loop dynamics. Coordination of Zn and Cu is then expected to have an opposing effect by constraining the loop, supported by the observation that the completely cysteine-depleted mutant elutes as a dimer in the holo state but monomerizes upon removal of the metals (Fig. 2).

The SOD Monomer Shows Low Stability and Slow Folding. The monomeric species produced by disulfide reduction of the apoSOD homodimer displays all of the kinetic and thermodynamic characteristics of fully structured subunits (Fig. 3). It folds cooperatively in a classical two-state reaction (27) that is overall indistinguishable from that of the structured monomer obtained by the interface mutations F50E/G51E (40). We note, however, that the stability and refolding rate constant of the reduced apoSOD^{wt} monomer are unusually low in relation to other single-domain proteins (Fig. 4). The equilibrium constant [D]/[M] at 0 M urea is $7/10^3$ (Table 1) compared, e.g., with $1/10^3$ to $1/10^7$ for the structurally analogous Ig-like β-sandwich proteins (42). With respect to the dynamics, reduced apoSOD^{wt} unfolds on average every 23 min and then refolds in 10 s. The corresponding lifetimes for the Ig-like β-proteins are 1–7 min and 0.004–0.7 s, respectively (42). For the oxidized apoSOD^{wt} monomer, [D]/[M] decreases to $5/10^4$, whereas the lifetimes of D and M change to 3 s and 108 min, respectively (Table 1). Accordingly, the refolding rate constant of the apoSOD monomer (0.1–0.3 s^{-1}) is among the lowest observed for single-domain proteins, in good agreement with results from theory (23). It is also much lower than expected from its value of the topological parameter "relative contact order" (0.15) that empirically correlates with the refolding rate constants across two-state proteins (43).

ϕ-Value Analysis. The ϕ-value of 1.0 for the substitution C6A implies that the structural environment of the thiol moiety consolidates early in the folding process and is close to native-like in the transition-state ensemble. The interactions involved are those that close up strands 1 and 8 in the β-barrel. The result agrees well with observations from simulations where this region is identified as important for two-state folding kinetics (23). Correspondingly, the low ϕ-value of 0.24 for the C111A mutant indicates that the loop between strands 6 and 7 is largely unstructured in the transition state. However, these results need to be verified by additional, more conservative mutations because the replacement C to A involves the truncation of a buried group with polar character that could be suspicious in ϕ-value analysis due to mismatched desolvation terms (25). A more reliable probe for the folding process is the C57–C146 disulfide linkage that yields a ϕ-value of 0.43. This result suggests that the large loop containing residues 49–84 is mainly disordered around position 57 in the transition state and attains its native structure after the rate-limiting step for folding. That is, the folding process of the SOD monomer is only marginally affected by the disulfide link. This finding is not surprising because the SOD monomer most likely needs to efficiently adopt its correct topological context to allow disulfide bond formation and metal incorporation in the reducing environment of the cytosol. The first folding step of SOD *in vivo* is thus likely to be the slow, cooperative formation of the relatively unstable disulfide-reduced monomer. In support of this conclusion, it was recently reported (40) that disulfide formation *in vivo* takes place in connection with copper loading of the folded SOD monomer through interactions with the copper chaperone (CCS).

Folding of the SOD Homodimer. Folding of the apoSOD homodimer reveals a unique kinetic fingerprint,

consistent with a simple three-state process (Scheme 2, where M is a high-energy intermediate at experimentally accessible protein concentrations (Fig. 5). A characteristic feature of the dimer chevron plot is that the folding process is rate-limited by the monomer formation under physiological conditions and that the transition state shifts to dimer dissociation at severely destabilizing conditions at high [urea], allowing full description of the parameters in Scheme 2. The apparent stability of the oxidized apoSOD^{wt}dimer, $\Delta G^{app}_{2D-M_2} = -RT\ln[D]/2[M_2]$, is $\approx$7 kcal/mol at 4 μM protein and 0 M urea, as estimated from equilibrium unfolding. For the holo protein, the corresponding value of $\Delta G^{app}_{2D-M_2}$ is $\approx$11 kcal/mol, as derived from data in ref. 30. The native SOD homodimer has thus a stability that falls in the typical range of other proteins (Fig. 4), possibly controlled by the *in vivo* requirements for efficient steady-state processing and degradation. It is then conceivable that the unusually low ΔG_{D-M} of the apoSOD monomer is an adaptation to maintain the native dimer at a biologically suitable stability.

Implications for Gain of Function. Taken together, the results in this study show that reduction of the C57–C146 disulfide bond is critical for the integrity of the dimer interface and shifts the equilibrium toward marginally stable monomers. For several of the ALS-associated mutations, these monomers even reach the critical point where they are fractionally unfolded at physiological conditions (M.J.L. and M.O., unpublished results). On this basis, the disulfide-reduced monomer stands out as an interesting candidate for noxious gain of function, either directly through excessive sampling of folding intermediates or unfolded conformations that are structurally promiscuous or, indirectly, through misfolding and aggregation (1,4,17, 18, 44–46). Consistently, the monomeric species has been implicated as a common precursor in SOD aggregation after oxidative damage of His and Phe side chains (47). Moreover, trapping of the dimeric state by the introduction of an intersubunit disulfide bond is reported to completely abolish aggregation of the A4V mutant that otherwise forms pore-like assemblies similar to those of other disease-associated proteins (4,44,48). The most compelling evidence that SOD molecules lacking the C57–C146 linkage are powerful inducers of cytotoxic function is found in the list of ALS-associated mutants. C146R and seven different C-terminal truncations (2) provoke disease although they miss one of the key thiol groups. The presence of disulfide-reduced SOD is also found to be the least common denominator in spinal-cord extracts from transgenic mice carrying the ALS-linked mutations G85R, D90A, G93A and the truncation variant G127insTGGG (S. Marklund, personal communication). An attractive feature of disease models linked to an increased load of monomeric protein is that they reconcile the structurally diverse set of mutations associated with ALS. Elevated levels of noxious monomers could arise from mutations that promote disulfide reduction (15), weaken the dimer interface (37), decrease the global stability (11–13, 47) or folding cooperativity (49) of either the monomeric or dimeric species, or adversely affect metal loading through the copper chaperone (CCS) (50). The low stability of the reduced monomer provides also an explanation for sporadic ALS. In these cases, impaired function of the cellular "housekeeping" system, i.e., chaperones, quality control, and degradation mechanisms, might cause the reduced form of wild-type SOD to enter the same cytotoxic pathway as the ALS-associated mutants, consistent with observations of cytosolic SOD-containing aggregates in both familial and sporadic ALS (18). Understanding the roles of the physiological disulfide-reduction systems in the pathology of SOD mutations and ALS is an obvious future goal.

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Footnotes

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Abbreviations: SOD, superoxide dismutase; apoSOD, SOD apoenzyme; ALS, amyotrophic lateral sclerosis.

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PNAS, July 12, 2005; 102(28): 9754 - 9759.

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Amyotrophic Lateral Sclerosis Mutations Have the Greatest Destabilizing Effect on the Apo- and Reduced Form of SOD1, Leading to Unfolding and Oxidative Aggregation

J. Biol. Chem., April 29, 2005; 280(17): 17266 - 17274.

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Small-molecule-mediated stabilization of familial amyotrophic lateral sclerosis-linked superoxide dismutase mutants against unfolding and aggregation

PNAS, March 8, 2005; 102(10): 3639 - 3644.

[Abstract] [Full Text] [PDF]

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I hereby certify that this paper, together with all enclosures identified herein, are being deposited with the United States Postal Service as first class mail, addressed to the Mail Stop Appeal Brief - Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450, on the date indicated below.

September 7, 2005
Date

Deborah A. Clark
Deborah A. Clark

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Art Unit	:	1651
Examiner	:	Francisco C. Prats
Appln. No.	:	10/018,770
Applicants	:	Yoshihito Ikeda et al.
Filing Date	:	December 17, 2001
Confirmation No.	:	2012
For	:	DRUG COMPOSITION CONTAINING A LECITHIN-MODIFIED SUPEROXIDE DISMUTASE

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

TRANSMITTAL OF APPEAL BRIEF
(PATENT APPLICATION - 37 CFR §41.37)

1. Transmitted herewith is the APPEAL BRIEF in this application, with respect to the Notice of Appeal filed on July 28, 2005.

2. STATUS OF APPLICANTS

This application is on behalf of:

___ other than a small entity.

_X_ a small entity.

A verified statement:

___ is attached.

_X_ was already filed.

3. **FEE FOR FILING APPEAL BRIEF**

Pursuant to 35 USC §41(a)(6), the fee for filing the Appeal Brief is:

X	small entity	$250.00
___	other than a small entity	$500.00
	Appeal Brief fee due:	$250.00

4. **EXTENSION OF TERM**

The proceedings herein are for a patent application and the provisions of 35 USC §41(a)(8) apply.

If an additional extension of time is required, please consider this a petition therefor.

(b) X Applicant believes that no extension of term is required. However, this conditional petition is being made to provide for the possibility that applicant has inadvertently overlooked the need for a petition and fee for extension of time.

5. TOTAL FEE DUE

The total fee due is: $250.00

Appeal Brief fee:	$250.00
Extension fee (if any)	$0
TOTAL FEE DUE:	$250.00

6. FEE PAYMENT

X Attached is a check in the sum of $250.00.

___ Charge Account No. 16 2463 the sum of $_____.

A duplicate of this transmittal is attached.

7. FEE DEFICIENCY

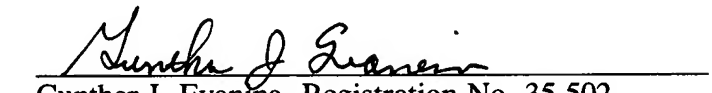
X If any additional extension and/or fee is required, this is a request therefor and to charge Account No. 16 2463.

and/or

X If any additional fee for claims is required, charge Account No. 16 2463.

Respectfully submitted,

September 7, 2005
Date



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